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Ph.D. Thesis – short version

Structural and molecular differentiation of sex chromosomes in Lepidoptera

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Declaration
I thereby declare that I prepared this thesis on my own, only with the use of relevant references.

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Annotation

Moths and butterflies (Lepidoptera) represent an excellent model for study of sex chromosome evolution. With my co-authors, we developed a straightforward method of collecting W-chromosome DNA by laser microdissection of sex-chromatin bodies from female polyploid cells of the codling moth, *Cydia pomonella*. The DNA was then used for creation of a W chromosome-specific plasmid library and preparation of the first chromosome painting probe in Lepidoptera. Similar probes were made from sex-chromatin of four representatives of Pyralidae and used for a comparison of similarity between their W chromosomes by means of the so called Zoo-FISH. Finally, a gross sequence composition of the W chromosomes in pyralids was probed by comparative genomic hybridization with male and female genomic DNAs. Our results revealed low similarity between the W chromosomes even in closely related species and degeneration of the W chromosomes via accumulation of repetitive sequences. Thus the results support the hypothesis of the accelerated molecular divergence of the lepidopteran W chromosomes in the absence of meiotic recombination.

Anotace

Motýli představují skvělý model pro studium evoluce pohlavních chromosomů. Spolu s mými spoluautory jsme vyvinuli metodu jak získat velké množství DNA z pohlavního chromosomu W pomocí laserové mikrodisekce pohlavních tělísek z polyploidních buněk samic obaleče jablečného, *Cydia pomonella*. Tato DNA byla použita pro výrobu plazmidové knihovny chromosomu W a pro přípravu první motýlí malovací sondy. Podobné sondy byly vyrobeny z pohlavních tělísek čtyř zástupců čeledi Pyralidae a použity pro srovnání vzájemné podobnosti jejich chromosomů W metodou Zoo-FISH. Nakonec jsme použili metodu komparativní genomové hybridizace samčích a samičích genomových sond pro hrubé srovnání sekvenčního složení chromosomů W zkoumaných druhů. Naše výsledky odhalily nízkou podobnost mezi chromosomy W, dokonce i u blízce příbuzných druhů, a degeneraci W chromosomů akumulací repetitivních sekvencí. Podporují tak hypotézu zrychlené molekulární divergence chromosomů W motýlů při absenci meiotické rekombinace.

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Co-authors' report of the contribution of Magda Vítková to the following publications:

Publication no. 1
Fuková I., Traut W., Vítková M., Nguyen P., Kubíčková S., Marec F. (2007) Probing the W chromosome of the codling moth, *Cydia pomonella*, with sequences from microdissected sex chromatin. *Chromosoma* **116**, 135-145. Epub 2006.

We declare that Magda Vitková contributed to this study significantly. Since the beginning she participated in the new strategy for sequence and evolutionary analysis of the lepidopteran W chromosome based on preparation of W-chromosome sequence libraries and painting probes by laser microdissection of sex chromatin. She participated namely in crucial experimental procedures including laser microdissection, DOP-PCR amplification and labelling of the W-chromosome painting probes, and Southern hybridization analysis of W-chromatin derived DNA clones.

Iva Fuková

Walther Traut

Petr Nguyen

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Publication no. 2
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We declare that Magda Vítková had a major contribution to this study. She prepared the W-chromosome painting probes by DOP-PCR amplification and labelling, made chromosome preparations from pyralid species under study, extracted genomic DNAs from the pyralids and prepared genomic probes, and designed and conducted all FISH, Zoo-FISH, and CGH experiments. She also carried out molecular cytogenetic analysis, prepared figures, and together with F. Marec prepared the manuscript.

Iva Fuková

Svatava Kubíčková

František Marec

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1 Introduction

1.1 Variants of the sex determination

Most eukaryotic organisms reproduce sexually to mix their genetic material and produce genetically diverse offspring. Decision whether the zygote will develop in a male or a female is made differently in different species. In principle, there are two ways. Either the primary sex determining signal is provided by environment (then the sex determination is called ESD) or it is determined genetically (GSD).

ESD in some taxa is controlled by temperature during embryonic development, typically in tuataras and crocodiles, but also in turtles, some lizards and fishes (reviewed in Ezaz et al. 2006, Mank et al. 2006). Sex can be determined also by means of social interactions with individuals of the same species. For instance, this has been proven in the fish bluehead wrasse, *Thalassoma bifasciatum*, where individually reared progeny develop in females, whereas those which are reared with other individuals developed in males (Munday et al. 2006). Similarly, in an echiuran worm, *Bonellia viridis*, larva develops in a male, if it has undergone a contact with a female, otherwise becomes a female itself (Jaccarini et al. 1983).

Haplodiploidy, a feature well known in Hymenoptera, but present also in Thysanoptera, some Homoptera, Coleoptera, and Acari (reviewed in Leather and Hardie 1995) is at the border between environmental and genetic sex determination. In this case, sex of the offspring is controlled by the mother, which produces diploid females when fertilizes the eggs or haploid males when does not.

Nevertheless, the overwhelming majority of eukaryotic organisms rely on genetic sex determination, where a gene or genes responsible for sex determination activate the sex determining pathway which results in development of the desired sex regardless of environmental conditions. These genes are localized on the sex chromosomes.

1.2 Sex chromosomes

1.2.1 Chromosomal sex determination systems

There are two main variants of chromosomal sex determination: XX/XY with heterogametic males and WZ/ZZ with heterogametic females. Both systems can occur also in their numerical variants including more than two sex chromosomes or, in contrast, variants Z0/ZZ or XX/X0 which miss the heteromorphic sex chromosomes. The system with heterogametic males is universal in mammals and most insects, whereas female heterogamety is typical for birds, snakes, caddis flies, and moths and butterflies (Ezaz et al. 2006, Marec and Novák 1998, Traut and Marec 1996). Some taxa (for instance fish and amphibians) possess both variants of chromosomal sex determination (Ezaz et al. 2006, Mank

et al. 2006). Finally, XX/XY and WZ/ZZ systems can occur in different populations of one species as in the Japanese frog *Rana rugosa* (Miura et al. 1998) or platy fish *Xiphophorus maculatus* (Kallmann 1968).

1.2.2 Mechanisms of sex chromosome evolution

Systems of chromosomal sex determination have evolved independently in many groups of animals and plants. Nevertheless, there are some common features, which suggest that sex chromosomes are products of similar evolutionary forces.

It has been repeatedly proven that sex chromosomes were originally homologous and had continuously diverged (Charlesworth 1996). The new sex chromosome diversification starts when one of the homologous partners acquires a gene (either by translocation of an old sex determining gene, SDG, from the old sex chromosome, or by alternation of one of its own genes) which short-circuit the old sex determination pathway. Since such locus will always or preferentially occur in one sex but never or less frequently in the latter, selection will prefer translocation or evolution of genes, the function of which is somehow connected with the respective sex, to the neighbourhood of this SDG (typically genes involved in spermatogenesis, if the SDG triggers development in a male). These genes are then linked to the SDG and, therefore, pass to the sex which uses them more. Since such alliance can only be broken by crossing-over, there will be a selective pressure to restrict the recombination between originally homologous chromosomes (e.g. via inversion). Level of restriction can range from over the sex determining region to the whole genome of heterogametic sex (for instance, *Drosophila* males and Lepidoptera females have no crossing-over) (reviewed in Charlesworth 1991; Marec 1996).

The restriction of recombination allows mechanisms leading to the Y chromosome erosion to take place. These mechanism are the 'Muller's ratchet', fixation of deleterious mutations on Y via 'hitchhiking' by favourable Y-linked mutations, and 'background selection'. The 'Muller's ratchet' is a term for stochastic loss of chromosomes carrying the lowest number of mutations. In recombining parts of the genome, chromosomes without mutations can be generated by means of the crossing-over. Once the crossing-over is suppressed, the number of mutant alleles will increase, since they only can be removed by a highly improbable reverse mutation (Charlesworth 1991).

The second mechanism shaping the Y and W chromosome is hitchhiking by favourable mutations. It is a common event depending on the linkage intensity between two genes, i.e. probability of crossing-over, so that it must occur on sex chromosomes where the recombination is usually restricted. All the genetic material on the Y (or W), including slightly deleterious mutations or elements, "hitchhikes" with genes essential for the sex determination and reproduction (Charlesworth 1996).

Finally, background selection is related to both previous mechanisms, because chance of a mutation to pass to the next generation depends on the neighbouring loci and the final

advantage/disadvantage score of the non-recombining region. Whereas highly deleterious mutations are removed by selection, weakly deleterious mutations are fixed (Bachtrog 2006).

Together with genes disabled by mutations, satellite sequences and transposons spread on the Y (or W) chromosome. Accumulation of self-replicating transposable elements is though to be enabled for several reasons. First, once the genetic activity of the Y (or W) is reduced there is a low probability of insertion into a transcriptionally active region and thereby its inactivation, which could lead to the carrier's death. Furthermore, recombination between similar sequences at different sites generates fatal chromosomal rearrangements and, therefore, their number is corrected by selection when crossing-over is present. Without recombination the transposable elements will tend to accumulate (Charlesworth 1991; Charlesworth et al. 1994).

Tandem repeats of simple DNA sequences (without transposition capabilities) also accumulate in regions of restricted exchange. They are generated by DNA self-replicative processes, and if there is a weak selection against individuals carrying high amount of selfish DNA, these tandem repeats of simple DNA sequences will equilibrate at much higher level than at the presence of recombination (Charlesworth 1991).

1.2.3 Sex chromosomes and age

All the aforementioned processes form the sex chromosome pair and diversify both their content and morphology. The evolution of sex chromosomes ends with a chromosomal pair of a gene-rich autosome-like X (or Z) chromosome and a degenerated Y (or W) chromosome, carrying only a few genes. In some cases, the heterogametic sex lacks the Y (or W) chromosome completely. Since the sex chromosome evolution starts over and over again, also the pairs of sex chromosomes display a different level of differentiation, which mostly reflects their evolutionary age. Hence, there is a scale of sex chromosome differentiation ranging from young homomorphic chromosomes, where sex chromosomes can not be distinguished from each other, to an old fully differentiated heteromorphic pair. Typical groups with many species possessing the homomorphic sex chromosomes are fishes and plants (Vyskot and Hobza 2004, Charlesworth et al. 2005). In contrast, neognathous birds are noted for their dissimilar sex chromosomes with a large Z chromosome and a small W chromosome (Ohno 1967).

Interestingly, different parts of the same heteromorphic sex chromosome can display different level of degeneration. This reflects different evolutionary strata of the chromosome, arisen from translocations or fusions with autosomes. These new parts of the sex chromosomes become subjects of the restriction of recombination and gradually degenerate, although the process is lagged compared to the older parts of the sex chromosome. This was observed, for instance, in the mammalian Y, chicken or *Silene* sex chromosomes (Bachtrog 2006, and references therein), or neo-W chromosomes in some subspecies of the silkmoth *Samia cynthia* and lymantriid moths *Orgyia thyellina* and *O. antiqua* (Yoshido et al. 2005).

Rate of the degenerative evolutionary processes was nicely demonstrated in studies on the sex chromosomes of flies. The neo-Y chromosome of *Drosophila pseudoobscura* and its relatives had evolved 13 Mya (million years ago) and appears to be fully degenerated (Carvalho and Clark 2005). In *D. miranda*, a piece of the chromosome 3 was translocated on the Y chromosome 2 Mya. Since that time this former part of an autosome has been inherited only from father to son and because there is no crossing-over in *Drosophila* males, it also stopped recombining. Despite this happened a short time ago (in evolutionary scale), genes on this new part of the neo-Y already display degeneration by mutations (Steinemann and Steinemann 1998). Comparison between human and chimpanzee MSY (male specific region on the Y) uncovered 16 genes common to both species. Although all of them are active in human, five of them are already non-functioning in chimpanzee. This means that they became pseudogenes during last 6 My (million years) after man and chimpanzee species separated (Hughes et al. 2005).

However, the Y and W chromosomes can fight against the degenerative mechanisms. The human Y chromosome has developed an extraordinary mechanism of self-renewing: the palindroms. Most of the genes localized on the male specific Y region are doubled, and their copies are organized in eight palindroms. If one of the copies is damaged by mutation, it still can be replaced by an unchanged copy by means of gene conversion. Therefore, gene copies localized in the palindroms display 99.9% similarity (Skaletsky et al. 2003). Extraordinary examples of long lasting genetic stability are the sex chromosomes Z and W in ratites. They both were shown to be almost completely homologous to each other and also nearly completely homologous to the chicken (*Gallus gallus*) Z chromosome. This actually means that the ratites maintained their sex chromosomes unchanged over 80 My (Shetty et al. 1999).

The mutation rate is different between the X and Y in general. The reason is (except a low efficiency of selection due to the degenerative processes mentioned above and low ability of reparation due to the lack of recombination) that the Y chromosome spends all its time in males where it has to undergo more cell divisions (and therefore it is more endangered by mutations) before it occurs in a sperm. Moreover, the sperm is an oxidative environment and lacks repair enzymes (Aitken and Marshall-Graves 2002). In contrast, the autosomes and the X chromosome spend half and two-thirds, respectively, of their lives in a female, which needs less cell divisions to produce an egg and provides more comfortable environment for the DNA. This was shown in human, where the Y displays a 4.8 times higher mutation rate than the rest of the genome. In addition, a comparison of the chimpanzee and human genomes revealed, that the most diversified chromosomes are the Ys, whereas the most similar are Xs. This finding nicely consists with the theory of different division number in males and females (The Chimpanzee Sequencing and Analysis Consortium 2005). However, the enhanced mutation rate is not shared with the W chromosome, which occurs solely in females (Marshall-Graves 2006, and reference therein).

1.3 Genes

1.3.1 Sex determining genes

Sex determination pathways and triggers have been studied in model organisms such as *Drosophila melanogaster*, *Caenorhabditis elegans*, mouse, and human. It seems that there are three models of the sex development activation. First, the presence of a dominant factor localized on the heteromorphic sex chromosome (Y or W) ultimately leads to the development of respective sex, regardless how many homogametic sex chromosomes are in the cell. Second, the dosage of a gene or genes localized on the homogametic sex chromosomes (X or Z) switches between the male and female developmental pathways (double dose leads to the homogametic sex development, whereas single chromosome gives rise to the heterogametic sex). Finally, the sex of a developing individual is chosen with the help of a gene product, which functions differently in different environmental conditions. The latter model represents the environmental sex determination (ESD, see above).

An example of the dominant sex determining factor in the heteromorphic sex chromosome is the sex determining gene *Sry* (Sex determining region on the Y chromosome). This factor was discovered in human, and it is a well conserved sex determining trigger in marsupial and eutherian mammals. As its name indicates, it is located on the Y chromosome, and its presence switches to the male development. Interestingly, its sequence seems to be poorly conserved among the mammals except its HMG box, a DNA binding domain, inherited from its progenitor, the *sox3* gene (reviewed in Waters et al. 2007).

An alternative mechanism of genetic sex determination consists in a gene (or genes) on the homomorphic sex chromosomes (X or Z), which switches the sexual development in a dosage-dependent manner. Such mechanism is supposed in birds, and their putative sex determining gene is *DMRT1* (doublesex- and mab-3-related transcription factor number 1) (Shan et al. 2000). A similar sex determination system involving besides the X-linked genes also autosomal elements is well known in the fruit fly *D. melanogaster*, where the sexual development is managed via genes placed both on the X chromosomes and autosomes. The cells "count" the autosome/sex chromosome ratio through interaction of products of these genes and consequently choose the correct sexual development (reviewed in Penalva and Sánchez 2003). In contrast to man, the fly Y chromosome plays no role in sex determination.

One would expect that so important feature such as sex determination is well conserved. However, the opposite is the truth. For instance, the gene *Sxl* (sex lethal) plays an important role in sex determination of *D. melanogaster* (Cline 1978). *Sxl* interprets the X:A ratio and passes the information to the next gene in the pathway, the *tra* (transformer) (Boggs et al. 1987). This function, however, holds only for Drosophilidae, whereas just non-drosophilid dipterans display different expression pattern of *Sxl*. This suggests that its role in these species is not sex determining. Actually, the *Sxl* sex determining role in drosophilids is an advanced feature, anticipated by a duplication of *Sxl* ascendant.

This occurred after the Brachycera clade had established and before the drosophilid species had diverged (Traut et al. 2006, and references therein).

In the medfly, *Ceratitis capitata*, the *tra* has even adopted the *Sxl* role and informs the cell about its sex status, although in this species the sex determining trigger is a dominant Y-linked gene (Pane et al. 2002). Finally, in the honey bee, *Apis mellifera*, the *tra* homologue *csd* became the primary sex determining factor (Beye et al. 2003).

It has been shown in *C. elegans* how easily the sex determining gene can be changed for another one. In mutant strains, various genes coding a transcription factor, a transmembrane receptor, a calpain-related protease, an extracellular protein, a conserved cytoplasmic protein, a phosphatase, a novel cytoplasmic protein, or tRNA were able to undertake the sex determining role (Hodgkin 2002, and references therein). Considering their variety, we can say that any gene can become the main sex determining trigger.

Even genes downstream from the main trigger display a low function homology. In the silkworm, *Bombyx mori*, homologs of some genes involved in the sex determination of *D. melanogaster* were found. However, except *dsx* (doublesex), which probably keeps its function even in vertebrates, other genes such as *tra/tra2* or *fru* (fruitless) seem to have another function than sex determination (Ohbayashi et al. 2002).

1.3.2 Genes on the sex chromosomes

The best studied heteromorphic sex chromosome nowadays is the human Y. It is much smaller than the X, being only 2–3% of the haploid genome, and is largely composed of repetitive sequences. It has two minor pseudoautosomal regions (PARs), homologous parts of the X and Y chromosomes, and a large non-recombining male specific region (MSY). The PAR1 is crucial for pairing of X and Y during male meiosis and its deletion leads to sterility. It is 2.6 Mb long and contains 24 genes, although in half of them their function remains unknown. In contrast, the PAR2 is only 320 kb long and carries 4 genes, two of them with a known function. It has been shown that defects of the PAR1/2-linked genes are responsible, for instance, for bipolar affective disorder or growth retardation and other skeletal abnormalities in Turner syndrome (reviewed by Mangs and Morris 2007).

The non-recombining MSY region is 23 Mb long and contains 78 protein-coding genes of three different origins: X-degenerated, X-translocated, and ampliconic. X-translocated genes are the least abundant (only two of them). They moved to the Y chromosome by simple transposition. X-degenerated genes are more frequent (16 genes); they are traces of former homology between the X and Y chromosomes. Finally, 60 ampliconic genes were acquired by the Y chromosome from different sources and multiplied. Interestingly, whereas the X-degenerated and X-translocated genes are expressed in various tissues, all the ampliconic genes are expressed in testes (Skaletsky et al. 2003).

Although the Y and W sex chromosomes are in general more altered in their sequence composition than their partners, the X and Z also change in respect of their genetic content. The reason

of increased sex biased genes on the Y and W is obvious: all genes in non-recombining region are linked to the sex-determining gene and, therefore, pass only to the sex which can exploit them. In contrast, the X- and Z-linked genes occur in both sexes and recombine regularly. However, they differ from the autosomes as well, since they spend two-thirds of their lives in the homogametic sex, compared to the autosome-linked genes, which occur in both sexes equally. A theory predicts that thanks to this fact the X and Z chromosomes tend to accumulate the sex-biased genes advantageous for the homogametic sex. In addition, in the heterogametic sex the X/Z-linked genes are hemizygous, i.e. lack their homologous partners, which gives a chance to all alleles to manifest themselves. This leads to a faster fixation of heterogametic sex-positive but recessive alleles. Therefore, the X and Z chromosomes should accumulate the male biased genes as well. Hence it has been suggested that both the male and female biased genes shall concentrate on the X and Z chromosomes (Rice 1984).

The only report on the sex-biased genes on the Z chromosome was obtained from chicken. It shows enrichment of the male-biased genes, while under-representation of female-biased genes (Storchova and Divina 2006). More experimental data are available on the sex-biased genes located on the X chromosomes. However, these data are rather inconsistent. The X chromosomes in species examined display either enrichment of female-biased genes (mouse) or random distribution throughout the genome (human, *C. elegans*). In contrast, male-biased genes are enriched in the human and mouse X chromosomes (Lercher et al. 2003, Khil et al. 2004), whereas in *C. elegans* and *D. melanogaster* are under-represented (Reinke et al. 2000, Parisi et al. 2003). The possible reason for male-biased gene paucity in the last case can be MSCI (Meiotic Sex-Chromosome Inactivation), a feature which takes place during meiotic division. During meiosis the sex chromosomes are temporarily heterochromatinized and transcriptionally silenced, whereas the other chromosomes remain active. Therefore, there is a selective pressure to move male-biased genes involved in male meiosis to autosomes, where they can function during the whole meiosis. This hypothesis is supported by experimental data from *D. melanogaster*, when genes involved in mitotic division prior meiosis in the male germ cells (which takes place before MSCI) are enriched on the X, whereas those which are expressed after MSCI during meiosis are preferentially located on the autosomes (see Bachtrog 2006, and references therein). However, male-biased genes expressed in somatic cells are also under-represented in *Drosophila* X chromosomes, which can not be explained by MSCI. Nevertheless, a mechanism responsible for this lack still remains unknown.

Feminization of the X chromosome can be managed either by means of translocations (i.e. physical movement of the female-biased genes onto the X or male-biased-genes onto the Y or autosomes), or by re-training of the X-linked genes with ubiquitous expression profile to a new female-specific/enriched function. Studies in mouse, human, and *Drosophila* revealed that genes with testes-specific expression tend to move out of the X chromosome to autosomes (Bachtrog 2006). Interestingly, the main mechanism of this exodus was reverse transcription and cDNA insertion of X-linked genes products, abusing reverse transcriptase of retrotransposons (Shiao et al. 2007).

Nevertheless, the very stable gene content in mammals and *Drosophila* suggests that the most of the sex-biased genes on the X chromosome arose via alternation of the X-linked gene function.

Experiments with *D. melanogaster* markers forcibly inherited only from mother to daughter revealed that any locus, which occurs in one sex only, would attract sex-biased genes advantageous for this sex even if they are detrimental for the other sex (Rice 1992). Moreover, this evolves very fast (29 generation in the Rice's study), suggesting that such mutations naturally occur in the gene pool, but in a low frequency.

1.4 The sex chromosomes of Lepidoptera

1.4.1 The origin of the W chromosome in Lepidoptera

As mentioned above, the male heterogamety heavily predominates in insects. The only known exception is the taxon Amphiesmenoptera, the superorder of two sister orders, the Lepidoptera (moths and butterflies) and the Trichoptera (caddis flies). In these orders females are the heterogametic sex with WZ or Z0 sex chromosomes and males are homogametic with ZZ sex chromosomes (Blackman 1995, Marec and Novák 1998). Other numerical variations of the WZ/ZZ sex chromosome system are known as well (Traut and Marec 1997; Traut 1999). Since the Z0/ZZ system is shared with Trichoptera and primitive Lepidoptera, the W chromosome absence seems to be a primitive feature. The W chromosome probably evolved in the common ancestor of Tischeriidae (a small taxon of leaf mining moths) and Ditrysia (the clade gathering 98% of all extant Lepidoptera species).

Two hypotheses have been proposed about the origin of the lepidopteran W chromosome. The first hypothesis suggests a fusion of the Z chromosome and an autosome; then the W chromosome has evolved consequently from the homologous partner of this autosome, giving rise of the neo-W and neo-Z chromosomes (Traut and Marec 1997). According to the second hypothesis, the function of the W chromosome was taken over by a B chromosome (Lukhtanov 2000).

1.4.2 Sex chromatin

Like the mammalian X chromosome, the W chromosome forms in female interphase nuclei a heteropycnotic body, called sex chromatin or W body. However, whereas the mammalian Barr body is a result of dosage compensation, a way of solving the double amount of genetic material in females in comparison with males, formation of the W body in Lepidoptera seems to be a way how the interphase cells get rid of the heterochromatinous W chromosome and move it aside transcriptionally active chromatin (Traut and Marec 1996). Nevertheless, unlike *Drosophila* which decreases amplification of the heterochromatin in polytene cells, Lepidoptera females amplify the whole genome including the heterochromatinous W chromosome. The W chromosome copy number in a polyploid cell in the

Malpighian tubes of *Ephestia kuehniella* was estimated to several thousands (Traut et al. 2007), while in *Bombyx mori* silk glands can reach even 4 x 10^5 (Gage 1974).

The presence of the W body in interphase cells can serve as a tool for quick determination of lepidopteran species which possess the W chromosome or, in those species which have the W, for sex determination in early ontogenetic stages (Traut and Marec 1996, 1997). Although the W chromosomes in most species form a single W body in the cells of most tissues, it has been shown that fusion of the W chromosome with an autosome or translocation of a Z-chromosome segment onto the W chromosome causes instability of the W body, which then disperses into more fragments (Traut et al. 1986, Marec and Traut 1994). This is probably caused by transcriptional activity of the autosomal or Z-chromosomal part. Morphology of the sex chromatin can therefore indicate a recent interchromosomal rearrangement involving the W chromosome.

Interestingly, 15 % of examined ditrysian species display no sex chromatin in either sex, whereas in 2 % of species the sex chromatin was present in both sexes. These data are interesting from the point of view of sex chromosome evolution, because ditrysian species without sex chromatin belong to different families, which always contain species with sex chromatin (Traut and Marec 1996). The W-chromosome absence must then be caused by several independent losses. It makes the Lepidoptera a beautiful demonstration of the rise and extinction of the W chromosome.

1.4.3 The W chromosome content

Using techniques of molecular cytogenetics it was shown in several species that the W chromosome considerably differentiated from its partner, the Z chromosome, by its genetic content even in those species, in which it was not recognized by its morphology. Available data suggest extraordinary high amount of repetitive elements, distributed throughout the whole genome, but accumulated on the W chromosome (Traut et al. 1999, Sahara et al. 2003a, Mediouni et al. 2004, Fuková et al. 2005). These findings were supported by data from sequencing of RAPD fragments derived from the *B. mori* W chromosome, in which truncated transposons heavily predominated, even in nested organization (reviewed by Abe et al. 2005). Although uncovering of DNA composition and consequent study of mechanisms of chromosome degeneration would be very interesting, obtaining this kind of data is extremely laborious. Repetitive elements of the W chromosome make sequence assemblage hardly feasible. This was the reason why Japanese and Chinese laboratories, which in parallel sequenced the genome of *B. mori*, used males to avoid problems with the W chromosome sequences (Mita et al. 2004, Xia et al. 2004).

In accordance with theories about sex chromosome evolution (degeneration in the case of the W chromosome) and experimental data from other taxa, the amount of genes on the lepidopteran W chromosome is very low. So far only four genes were proposed to be located on the lepidopteran W chromosomes, but most of them have not been isolated or sequenced. Moreover, only two of the genes are connected with the sex control or function.

The first W-linked gene is the master trigger of sex determination, a feminizing factor *Fem*. Already Hasimoto (1933) concluded that the sex of the silkworm, *Bombyx mori*, is controlled by the presence/absence of the W chromosome. This conclusion was based on data obtained by means of cross-hybridization experiments with triploid (ZZW+3A) and tetraploid (ZZWW+4A) silkworms, where all animals possessing the W chromosome developed ultimately in females, regardless of a different ratio of the Z chromosomes and autosomes. The putative dominant W-linked female determining factor was named *Fem* (feminizing factor). Although its presence has been proposed a long time ago, the gene itself remains unknown despite intensive research effort. Nevertheless, very recently H. Abe and his colleagues studied several *B. mori* strains with different W-chromosome deletions using RAPD markers and localized the putative *Fem* near the W-Rikishi marker, which made the *Fem*-containing region much narrower (Abe et al. in Iatrou and Couble 2007). Moreover, last year one Japanese group and one Indian group independently isolated W-linked zinc-finger genes by means of differential display and subtraction, respectively. The genes seem to be expressed in early embryo during the time when sex determination takes place (Ajimura et al. and Satish et al., both in Iatrou and Couble 2007). However, further analyses are required to confirm their sex determining function.

The second W-linked gene, discovered by N. Kawamura by means of ZZW and ZWW triploid crosses, is *Esd* (Egg size determining gene), later found to be identical with the gene *Ge* (Giant eggs) (Kawamura 1988, 1990). An extraordinary case of the W-linked gene is *per* (period), a gene involved in circadian rhythms. The *per* gene is located on the Z chromosomes of *Antheraea pernyi*, but possess multiple copies also on the W chromosome of *A. pernyi* (Gotter et al. 1999). At least some of them are active, although their function was probably altered. Finally, results obtained from sex chromosome mutants of *E. kuehniella* suggest the presence of a male-killing factor in the W chromosome (Marec et al. 2001).

1.4.4 The Z chromosome content

The most complex information about the lepidopteran Z chromosome was provided by Koike et al. (2003), who sequenced and analyzed a 320 kbp long contig derived from *B. mori* Z chromosomes. In this region, they discovered 14 genes, 47 non-LTR retrotransposons, 50 retroposons, 10 DNA-type transposons, and other uncharacterized repetitive sequences, but no LTR retrotransposons. Interestingly, all retrotransposons and DNA-type transposons were incomplete, in contrast to the W chromosome which possesses a high amount of the complete transposable elements (see Koike et al. 2003, and references therein). Synteny mapping of isolated genes between *B. mori*, *D. melanogaster*, and *C. elegans* revealed a poor gene order-conservation. The only exception is a conserved cluster of three genes present in all three species (Koike et al. 2003). Nevertheless, synteny mapping in lepidopteran species shows highly conserved gene order in autosomes and the Z chromosome not only between members of related families Bombycidae (*B. mori*) and Sphingidae (*Manduca sexta*), but also

between rather evolutionary distant species such as *B. mori* and *Heliconius melpomene* (Nymphalidae), which diverged 106 Mya (Yasukochi et al. 2006, Pringle et al. 2007, Sahara et al. 2007).

1.4.5 Sex determination in Lepidoptera

As mentioned above, the sex determining gene in *B. mori* is the feminizing factor *Fem*, but its sequence is not yet known. Besides *B. mori*, almost no information is available about the sex determination trigger in Lepidoptera. The only fact, which is obvious, is that species lacking the W chromosome have to rely on a Z-linked dosage dependent gene or genes. However, even the species of advanced Lepidoptera, which possess the W, can rely on their Z with respect to sex determination. For example, a psychid moth *Solenobia triquetrella* is such a candidate for the Z-linked dosage dependent sex control. In this species, crosses between females of a natural tetraploid parthenogenetic population and males of a diploid bisexual population generated intersex offspring (reviewed by Traut et al. 2007, and references therein).

As already mentioned, homologs of the genes *dsx* (doublesex), *tra* and *tra-1* (transformer) expressed downstream in the *Drosophila* sex determination pathway were found in the *Bombyx* genome. Whereas the *Bombyx dsx* due to its sex-specific alternative splicing resembles its *Drosophila* homolog, function of *tra* and *tra-1* seems to be different (Ohbayashi et al. 2002).

1.5 Cytogenetic tools for studying the sex chromosomes

1.5.1 "Classical" cytogenetics

In those lepidopteran species which possess "old" sex chromosomes, the W chromosome is usually degenerated and heterochromatinized enough to be easily visualized by means of pachytene mapping, using a simple staining with orcein and phase contrast microscopy (Traut and Marec 1997). In the pachytene stage, the W and Z chromosomes pair and form a regular bivalent. In the WZ bivalent, the heterochromatinous W chromosome forms a conspicuous and mostly continuous thread, in contrast to the other chromosomes (including the Z chromosome) which display a regular pattern of chromomeres (Marec and Traut 1994). In some species such as the codling moth, *Cydia pomonella*, the W chromosome can be identified even in mitotic metaphases, where the chromosomes are small and appear homogeneous, after staining with DAPI (F. Marec, unpublished), a fluorescent dye which highlights regions of heterochromatin rich in A-T base pairs. Nevertheless, these methods are inapplicable in species with young sex chromosomes (for instance, in two *Orgyia* species with a neo-W/neo-Z sex chromosome system; Traut and Marec 1997, Yoshido et al. 2005) and also in some species as the wax moth, *Galleria mellonella*, possessing a well differentiated W chromosome, which,

however, is distinguishable only in postpachytene nuclei of nutritive cells, whereas is undetectable in oocytes and early pachytene in general (Vítková et al. 2007).

1.5.2 Fluorescence in situ hybridization with genomic probes

Advanced techniques, modifications of fluorescence in situ hybridization (FISH), such as genomic in situ hybridization (GISH) and comparative genomic hybridization (CGH) are based on co-hybridization of one (GISH) or two (CGH) differently labelled genomic probes in the presence of species-specific competitor (Cot-1 DNA or surplus of sonicated genomic DNA of the homogametic sex). With these techniques, the W chromosome can be distinguished by its genetic content even in species, where the W cannot be identified by its morphology with conventional cytogenetic methods. Traut et al. (1999) used CGH for the study of molecular differentiation between sex chromosomes in three lepidopteran species (*B. mori*, *E. kuehniella*, and *G. mellonella*), two flies *D. melanogaster* and *Megaselia scalaris*, man, and a mouse. Genomic probes differentiated the Ws of all three moths and the Y chromosome in man, mouse, and *Drosophila*, but not the young sex chromosomes in *Megaselia*. These results suggested the evolutionary youth of *Megaselia* sex chromosomes, which did not have enough time to diverge at the morphology or sequence levels.

Whereas GISH can simply visualize the W chromosome, the comparison of male and female genomes with CGH brings additional information about the rough sequence composition of the W chromosome. A comparison of hybridization signals of male and female genomic probes suggested that the W chromosome is composed of two kinds of DNA sequences (Sahara et al. 2003a). These were (i) female specific sequences, present only in the W chromosome, and (ii) ubiquitous sequences, present elsewhere in the genome but accumulated in the W chromosome. Interestingly, some species seem to possess uniquely ubiquitous sequences, whereas the others have both sequence types (Sahara et al. 2003a).

1.5.3 Chromosome painting and Zoo-FISH

Alternatively, sex chromosomes can be visualized and studied by means of FISH with the sex-chromosome specific painting probes. In vertebrates, painting probes became a mighty tool in chromosome homology searches by cross-hybridizations (e.g. Huang e al. 2006); they are also important in human and veterinary medicine (e.g. Hernando et al. 2002, Iannuzzi et al. 2001). One of the remarkable reasons of today's availability of vertebrate painting probes is a big size of metaphase chromosomes, together with the presence of various markers such as G-bands, centromere position, and length differences. To prepare such probes the chromosomes can be either flow-sorted or microdissected in sufficient numbers for further analysis. However, the chromosomes in Lepidoptera are numerous, small, uniform in length and holokinetic (lacking centromere). In addition, G-banding does not work in Lepidoptera as well as in many other invertebrates. These characteristics make

identification of individual chromosomes difficult. An exception is the W chromosome formed largely of heterochromatin, which makes it recognizable in some species but only in the pachytene stage, in which it cannot be separated from its pairing partner.

1.5.4 Bacterial artificial chromosomes (BACs)

Whereas the chromosome sorting or microdissection are hardly feasible in Lepidoptera and also highly exceptional in other invertebrates, the BACs (Bacterial Artificial Chromosomes) are widely used tools. They consist of a vector based on the *Escherichia coli* F-plasmid which is able to carry an 80-300 kbp long fragment of genomic DNA of a particular organism (Shizuya et al. 1992). Advantages of BACs consist in the size of their inserts and easy handling of the BAC library. The BAC inserts are by far long enough to allow the use of BACs directly as probes for FISH and, therefore, localization of a particular BAC on the chromosome by the so-called BAC-FISH. In addition, they are handled as any DNA library, including easy selection by means of filter hybridization and consequent multiplication of selected BACs in bacteria. Finally, the size of the inserts is still available for sequencing, which makes possible the identification of sequences adjacent to the marker.

For example, BAC clones were used as markers for identification of all chromosomes including the W chromosome in *B. mori* (Sahara et al. 2003b, Yoshido et al. 2005) or for the identification of conserved synteny between *B. mori*, *H. erato*, and *M. sexta* (Yasukochi et al. 2006, Sahara et al. 2007). On the other hand, BACs seem to be highly inefficient in Zoo-FISH, a cross-hybridization of a BAC probe from one species to chromosome spreads of another species. This was first shown by Yoshido et al. (2007), who used the W-derived BAC probes with the aim to identify homologous regions between the W chromosomes of different species. However, the probes failed to hybridize to the W chromosomes in any lepidopteran species except *Bombyx mandarina*, the wild ancestor of *B. mori*. An easy explanation of this feature is an extraordinary amount of repetitive sequences on the W chromosome. Such "junk" DNA evolves quickly, and its similarity is supposed to decrease rapidly among species. However, the Z-derived probes also failed to detect their homologous loci in other species, in spite of euchromatin prevalence (F. Marec, personal communication). Hence, for synteny mapping it is necessary to select the corresponding BACs from respective species and compare their localization and order on the chromosomes by "species-specific" BAC-FISH. The possible reason of hybridization failure could be presence of a high amount of interspersed repetitive DNA. This hypothesis is supported by a necessity of use a high amount of unlabelled male DNA as a competitor in the probe cocktail. Without competitor the hybridization signals of BAC probes are not enough specific (F. Marec, personal communication). Finally, hybridization signals of W-derived BACs are special. Whereas autosome- or Z-derived BACs generate single dot-like hybridization signals, the W-derived BACs provide similar hybridization pattern as genomic probes in GISH or CGH, i.e. the signals are not localized to a particular region but highlight the entire W chromosome.

This suggests uniform distribution of similar repetitive sequences and transposons in the W chromosome (Sahara et al. 2003b).

1.6 Why to study the sex chromosomes of Lepidoptera?

The Lepidoptera is one of the largest animal taxa, gathering more than 130, 000 of known species. Their ancestral sex-chromosome determination system is Z0/ZZ. This system appears conserved in several basal families, whereas the overwhelming majority of species have a WZ/ZZ system including its numerical variants (multiple sex chromosomes), neo-Z chromosomes, neo-W chromosomes as well as secondary losses of the W chromosome. These features reflect different stages of the sex chromosome differentiation, and their study can help us to understand the basic mechanisms of sex-chromosome evolution. Here I present our contribution to this understanding.

2 Outline of research

Sex chromosomes represent an outstanding genetic material, which is mastered by different rules than the rest of the genome. The occurrence of sex determining genes, consequent restriction of recombination and different time, which each sex chromosome spends in a female or a male, cause anomalous selective pressures which has led to the alteration of the sex chromosome genetic content. The Lepidoptera, represent a perfect model group for studying these mechanisms. They show a variety of sex chromosomes systems ranging from the primary Z0/ZZ, over advanced WZ/ZZ including derived multiple sex chromosomes to the secondary Z0/ZZ system. Thus, among systems with female heterogamety only Lepidoptera display the full evolutionary life cycle of the W chromosome, from its beginning through molecular and structural differentiation, rearrangements with autosomes and eventual loss. The aim of my thesis was to contribute to uncovering the evolutionary forces shaping the lepidopteran W chromosome by studying its structure and molecular composition and by comparative analysis of its interspecific similarity and/or divergence.

In the present papers, we have developed a new technique for the study of lepidopteran W chromosomes. In selected species with well differentiated sex chromosomes, we collected a high number of W-chromosome copies by laser microdissection of the sex chromatin bodies from highly polyploid female cells. Thus, we obtained relatively large samples of W-chromatin DNAs, which were further used for (i) generating a W-chromosome sequence library and (ii) preparation of W-chromosome specific painting probes. These tools were then used in two different studies, which are detailed in Chapter One and Chapter Two, respectively.

In chapter one I present a work which motivation was obtaining the W-chromosome markers, which could be consequently used for a detailed analysis of the W-chromosome composition and for sexing of codling moth, *C. pomonella*. We took advantage from the feature common for all W-positive lepidopteran species, the sex chromatin. We isolated sex chromatin from polyploid cells of Malpighian tubes by laser microdissection, amplified by means of DOP-PCR (degenerated oligonucleotide-primed PCR) and used for developing the first lepidopteran painting probe and making the W-chromosome library. The W fragments in the library were sequenced and analyzed by Southern blot and homology search via BLAST and Kaiko-BLAST.

In chapter two I present a work where we used the laser microdissection of sex chromatin to prepare the W-painting probes from four representatives of Pyralidae with the aim to compare the similarity of their W chromosomes. Whereas all four probes were used for differentiation of the W in corresponding species, the W-painting probe of one species was hybridized to all four pyralids to reveal homologous regions. Then we performed CGH in all species under study to compare the ratio and distribution of the W-specific/ubiquitous sequences on their W chromosomes. From specificity of W-painting probe hybridization signals and results of CGH we concluded the nature of W-linked sequences in tested species.

3 Original publications

3.1 Chapter one:

Probing the W chromosome of the codling moth, *Cydia pomonella*, with sequences from microdissected sex chromatin

Iva Fuková, Walther Traut, Magda Vítková, Petr Nguyen, Svatava Kubíčková and František Marec
Chromosoma **116**: 135-145 (2007)

Abstract

The W chromosome of the codling moth, *Cydia pomonella*, like that of most Lepidoptera species, is heterochromatic and forms a female-specific sex chromatin body in somatic cells. We collected chromatin samples by laser microdissection from euchromatin and W-chromatin bodies. DNA from the samples was amplified by degenerate oligonucleotide-primed polymerase chain reaction (DOP-PCR) and used to prepare painting probes and start an analysis of the W-chromosome sequence composition. With fluorescence in situ hybridization (FISH), the euchromatin probe labelled all chromosomes, whereas the W-chromatin DNA proved to be a highly specific W chromosome painting probe. For sequence analysis, DOP PCR-generated DNA fragments were cloned, sequenced, and tested by Southern hybridization. We recovered single copy and low-copy W-specific sequences, a sequence that was located only in the W and the Z chromosome, multicopy sequences that were enriched in the W chromosome but occurred also elsewhere, and ubiquitous multi-copy sequences. Three of the multi-copy sequences were recognized as derived from hitherto unknown retrotransposons. The results show that our approach is feasible and that the W-chromosome composition of *C. pomonella* is not principally different from that of *Bombyx mori* or from that of Y chromosomes of several species with an XY sex determining mechanism. The W chromosome has attracted repetitive sequences during evolution but also contains unique sequences.

3.2 Chapter two:

Molecular divergence of the W chromosomes in pyralid moths (Lepidoptera)

Magda Vítková, Iva Fuková, Svatava Kubíčková and František Marec

Chromosome Res **15:** 917-930 (2007)

Abstract

Most Lepidoptera have a WZ/ZZ sex chromosome system. We compared structure of W chromosomes in four representatives of the family Pyralidae - *Ephestia kuehniella*, *Cadra cautella*, *Plodia interpunctella*, and *Galleria mellonella* - tracing pachytene bivalents which provide much higher resolution than metaphase chromosomes. In each species, we prepared a W-chromosome painting probe from laser-microdissected W-chromatin of female polyploid nuclei. The *Ephestia* W-probe was cross-hybridized to chromosomes of the other pyralids to detect common parts of their W chromosomes, while the species-specific W-probes identified the respective W chromosome. This so-called Zoo-FISH revealed a partial homology of W-chromosome regions between *E. kuehniella* and two other pyralids, *C. cautella* and *P. interpunctella*, but almost no homology with *G. mellonella*. The results were consistent with phylogenetic relationships between the species. We also performed comparative genomic hybridization, which indicated that the W chromosome of *C. cautella* is composed mainly of repetitive DNA common to both sexes but accumulated in the W chromosome, whereas *E. kuehniella*, *P. interpunctella*, and *G. mellonella* W chromosomes also possess a large amount of female specific DNA sequences, but differently organized. Our results support the hypothesis of the accelerated molecular divergence of the lepidopteran W chromosomes in the absence of meiotic recombination.

4 Summary of results and discussion

In chapter one I presented a work, where we collected W-chromosome DNA by laser microdissection of sex-chromatin bodies from female polyploid cells of the codling moth, *Cydia pomonella*, and amplified by degenerate oligonucleotide-primed PCR (DOP-PCR). Then one DNA sample was used to prepare a W-chromosome painting probe by secondary DOP-PCR amplification and labelling with a fluorochrome. Using FISH we showed that the W-chromatin derived probe exhibits a high specificity and paints the whole W chromosome. Once the specificity of the probe was confirmed we created a W chromosome-specific plasmid library by cloning another DNA sample. More then 50 clones were sequenced. The sequence analysis revealed 17 DNA motifs, out of which three motifs showed a significant homology to retrotrasposons known from *Drosophila melanogaster* and *Bombyx mori*, while the remaining 14 motifs represented unknown sequences. The motifs were further tested by Southern hybridization to male and female genomic DNAs, and consequently divided into four categories: (1) female-specific single-copy and low-copy sequences, i.e. sequences localized exclusively in the W chromosome; (2) a low-copy sequence occurring in both sexes but located only in the W and Z chromosomes; (3) multi-copy sequences that are highly enriched in the W chromosome but occur also elsewhere; (4) ubiquitous multi-copy sequences predominantly occurring in autosomes but also present in the W chromosome. Overall, our results suggest an abundance of highly repetitive sequences in the W chromosome, which supports the hypothesis formulated earlier on the basis of CGH results in several lepidopteran species (Traut et al. 1999, Sahara et al. 2003a, Fuková et al. 2005). Nevertheless, the codling moth W chromosome still contains some single- and low-copy W-specific sequences, which may serve as molecular markers of the W chromosome and/or female sex. The sequence data obtained in this study represent the second largest survey on the molecular composition of the W chromosome in Lepidoptera (after the silkworm, *B. mori*; Abe et al. 2005). Finally, the W-painting probe developed in the codling moth represents the first chromosome-specific painting probe prepared by laser microdissection not only in Lepidoptera but in all insects and most probably also in all invertebrates.

In chapter two I presented a work, where W-chromosome painting probes prepared by laser miscrodissection of sex chromatin from four moths of the family Pyralidae were used to test similarity of their W chromosomes by cross-hybridizations, i.e. by comparative chromosome painting (the so-called Zoo-FISH). The species were the flour moth (*Ephestia kuehniella*), almond moth (*Cadra cautella*), and Indian meal moth (*Plodia interpunctella*) from the subfamily Phycitinae, and the wax moth (*Galleria mellonella*) from the subfamily Galleriinae. This approach showed a considerable sequence divergence between the W chromosomes of even closely related species from the same

subfamily Phycitinae, and revealed almost no homology between *E. kuehniella* and *G. mellonella*, belonging to different subfamilies. Interestingly, the W-painting probes hybridized not only to the W chromosome, but also to the other chromosome, though less intensely. This suggested the presence of ubiquitous sequences accumulated on the W chromosome. With the aim to obtain more information about these sequences, we used CGH with male and female genomic probes. CGH highlighted regions of female specific sequences in *E. kuehniella*, *P. interpunctella*, and *G. mellonella*, whereas in *C. cautella* revealed the W chromosome content composed largely of ubiquitous sequences. However, CGH with directly labelled probes has limits in respect of sensitivity. Therefore, it is not possible to exclude the presence of W-specific sequences in *C. cautella* W chromosome. On the other hand, it is obvious that their amount, if they are present, is incomparably lower then in the other three species. Moreover, distribution of both sequence types differed in particular species. In *G. mellonella* and *P. interpunctella*, the female specific sequences were concentrated in one region, while they were interspersed in *E. kuehniella*.

The W chromosome comparison by means of Zoo-FISH revealed a low sequence homology even in representatives of the same subfamily, and almost none between subfamilies. This feature reflects the independent evolution of the W chromosomes in Lepidoptera, where each W chromosome undergoes stochastic changes driven by degenerative mechanisms occurring in every non-recombining part of the genome. The results of sequence analysis of *C. pomonella* W-derived clones, FISH with W-painting probes and CGH in pyralids suggest preponderance of repetitive sequences present elsewhere in the genome, but accumulated on the W chromosome. Their character can be hypothesized from sequenced parts of the *B. mori* W chromosome, where transposable elements heavily predominate (reviewed by Abe et al. 2005), and also from our genomic survey of the codling moth W chromosomes, where sequences derived from retrotransposons were found. These data supports the theory of Charlesworth (1991) that mobile elements are the main evolutionary tool of the Y/W chromosome degeneration.

5 Future plans

We showed that sequencing of microdissected W-bodies is an excellent tool for getting a general overview of sequences on the W chromosome (Fuková et al. 2007; see Chapter One). With CGH we revealed considerable differences in the ratio between W-specific and ubiquitous sequences on the W chromosomes of pyralid species but also a great diversity in their distribution (Vítková et al. 2007; see Chapter Two). In future, I would like to perform a genomic survey of the pyralid W chromosomes using cloning and sequencing of microdissected sex chromatin of *Plodia interpunctella*, *Cadra cautella*, and *Ephestia kuehniella* with the aim to compare W-chromosome homology at the sequence level and uncover character of the W-specific sequences. Further, our Zoo-FISH experiments with the W-painting probes suggested a relatively fast sequence divergence of the W chromosomes of pyralids (Vítková et al. 2007; see Chapter Two). However, our knowledge about the evolutionary time of their divergence is vague and, therefore, we can not make a general conclusion about the rate of molecular evolution of the non-recombining W-chromosome in comparison with other chromosomes. This can be solved by parallel cross-hybridization of Z-chromosome painting probes to characterize the decrease of sequence homology in relation to the increasing evolutionary distance between the species and compare data obtained with those from Zoo-FISH with W-paining probes. Therefore, we work on developing Z-painting probes. Finally, I intend to repeat the Zoo-FISH experiments with W-painting probes in species from another lepidopteran taxon such as, for example, the family Nymphalidae, where the evolutionary relationship and time of divergence is known (Wahlberg 2006).

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